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Acetylcholine Receptor Clustering Is Triggered by a Change in the Density of a Nonreceptor Molecule

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Abstract. Acetylcholine receptors become clustered at the neuromuscular junction during synaptogenesis, at least in part via lateral migration of diffusely expressed receptors. We have shown previously that electric fields initiate a specific receptor clustering event which is dependent on lateral migration in aneural muscle cell cultures (Stollberg, J., and S. E. Fraser. 1988. *J. Cell Biol.* 107:1397–1408). Subsequent work with this model system ruled out the possibility that the clustering event was triggered by increasing the receptor density beyond a critical threshold (Stollberg, J., and S. E. Fraser. 1990. *J. Neurosci.* 10:247–255). This leaves two possibilities: the clustering event could be triggered by the field-induced change in the density of some other molecule, or by a membrane voltage-sensitive mechanism (e.g., a voltage-gated calcium signal).

Electromigration is a slow, linear process, while voltage-sensitive mechanisms respond in a rapid, nonlinear fashion. Because of this the two possibilities make different predictions about receptor clustering behavior in response to pulsed or alternating electric fields. In the present work we have studied subcellular calcium distributions, as well as receptor clustering, in response to such fields. Subcellular calcium distributions were quantified and found to be consistent with the predicted nonlinear response. Receptor clustering, however, behaves in accordance with the predictions of a linear response, consistent with the electromigration hypothesis. The experiments demonstrate that a local increase in calcium, or, more generally, a voltage-sensitive mechanism, is not sufficient and probably not necessary to trigger receptor clustering. Experiments with slowly alternating electric fields confirm the view that the clustering of acetylcholine receptors is initiated by a local change in the density of some nonreceptor molecule.

One of the central events in synaptogenesis is the localization of specific molecules at the postsynaptic membrane. The neuromuscular junction is a well studied example in which acetylcholine receptors (AChRs),[1] acetylcholinesterase, basal lamina components, and numerous cytoskeletal elements are concentrated on the muscle surface contacted by the motor neuron (reviewed in Schuetze and Role, 1987). The clustering process begins within hours of neuronal contact, but the stability of receptor clusters continues to increase over a period of days to weeks. This suggests that receptor clustering may involve multiple mechanisms acting over the time course of synaptogenesis, but the specific mechanisms involved are as yet unresolved. Early in the clustering process, AChRs are concentrated at least in part by the lateral migration and aggregation of diffusely distributed receptors (Anderson, 1977; Frank and Fischbach, 1979; Kuromi and Kidokoro, 1984; Ziskind-Conhaim et al., 1984; Role et al., 1985). It is these earliest events in receptor localization that are addressed in the experiments presented here.

Electric fields can be used to induce the redistribution of membrane components on the cell surface (Jaffe, 1977; Poo, 1981; McLaughlin and Poo, 1981). Such fields have also been demonstrated to initiate an AChR clustering event on spherical muscle cells (myospheres) in culture (Poo, 1981; Stollberg and Fraser, 1988). That is, AChRs aggregate at the cathode-facing cell pole in response to a field, and continue to aggregate after the field has been terminated. This experimental system has several attractive features for studies of receptor clustering mechanisms. The clustering event is at least somewhat specific for AChRs in that Con A binding sites are not clustered (Stollberg and Fraser, 1988). The cluster-inducing stimulus can be precisely controlled, and the density of receptors on the cell surface can be readily quantified. Finally, the rapid response of the system and the temporal resolution of the analysis permit study of the earliest events in receptor clustering.

There are only three known mechanisms by which electric fields could initiate receptor clustering (Stollberg and Fraser, 1990). In the first scenario, the field induces electromigration of AChRs toward the cathodal pole, thereby causing a

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1. *Abbreviation used in this paper*: AChR, acetylcholine receptor.

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local increase in receptor density. Once the density of AChRs exceeds some threshold, receptor clustering is triggered. A second possibility is similar except that it entails the field-induced concentration of non-AChR molecules as the triggering event which initiates receptor clustering. Finally, receptor clustering could be triggered by a change in the transmembrane voltage potential. At the cathode-facing pole, where receptor aggregation occurs, the cell membrane is depolarized by ~10–15 mV under the conditions reported previously. Thus, a voltage-sensitive mechanism (e.g., a voltage-gated calcium channel) might be locally activated, thereby initiating receptor clustering.

The first possibility, that AChR clustering is triggered by the local increase in receptor concentration, was ruled out by finding conditions under which receptor migration proceeded toward the anodal pole, while receptor aggregation continued at the cathodal pole (Stollberg and Fraser, 1989, 1990). This leaves only the latter two possible mechanisms, termed the "electromigration" and "voltage-sensitive" hypotheses, respectively. Here we demonstrate that receptor clustering is not triggered by a rise in intracellular calcium, nor by a mechanism with the response characteristics of some other voltage-sensitive mechanism. Instead, receptor clustering behavior appears to be triggered by the field-induced accumulation of some non-AChR molecule(s).[2]

Materials and Methods

Culture System

Embryonic muscle cells were dissected from stage 18–20 *Xenopus laevis* embryos (Nieuwkoop and Faber, 1962) in Steinberg's solution (58.2 mM NaCl, 0.7 mM KCl, 0.4 mM $Ca(NO_3)_2$, 4.6 mM Tris, 50 μg/ml gentamycin), pH 7.8, containing 1 mg/ml collagenase. Cells were dissociated by incubation for 3–5 min in Ca^{++} free Steinberg's solution (containing 0.3 mM EDTA) followed by mild trituration. The cultures were maintained on sterile coverslips in drops of culture medium (85% Steinberg's solution, 10% Leibovitz's L-15 medium, 5% fetal calf serum, pH 7.8) for 1 d at 24°C.

Electric Field Application

Cultures were assembled into electric field chambers with internal dimensions of 6.0 × 1.0 × 0.02 cm deep (AChR experiments) or 4.0 × 1.0 × 0.02 cm deep (fura-2-experiments). Electric fields were applied to the chambers with 6 mm (inside diameter) glass tubes filled with 2% agar/Steinberg's solution. Agar bridges made from PE90 tubing were used to monitor the voltage drop (and hence the applied field strength) across the chambers.

Voltage potentials were generated by an electrophoresis power supply (model No. 493; Instrumentation Specialties Company, Lincoln, NE). Alternating fields were applied by switching this source through a double-throw, double-pole relay (STO40-1; General Electric Company, West Lynn, MA) driven by an SD9 stimulator (Grass Instrument Co., Quincy, MA). A 50-kΩ potentiometer in series with one of the relay outputs was used to adjust differentially the field strength in the two phases of alternating electric fields. The fields were monitored on an oscilloscope (model 535A; Tektronix, Inc., Portland, OR).

Two rapidly alternating field paradigms were used, both of which consisted of repeated, contiguous trains of a 100-ms waveform (see Fig. 1). In the first paradigm the alternating field was symmetric, consisting of 50 ms of the field in one orientation followed by 50 ms of the same field strength in the reverse orientation (Fig. 1, *A* and *B*). In the second paradigm the field was asymmetric with respect to field strength and duration. This paradigm consisted of a 10-ms field application followed by 90 ms of one-ninth of the original field in the reverse orientation (Fig. 1 *C*). For both symmetric and asymmetric alternating fields the average field strength is zero ($[field_1 \cdot duration_1] + [field_2 \cdot duration_2] = 0$).

2. Some of this work has been presented in abstract form (Stollberg, J., and S. E. Fraser. 1989. *Soc. Neurosci. Abstr.* 70.8).

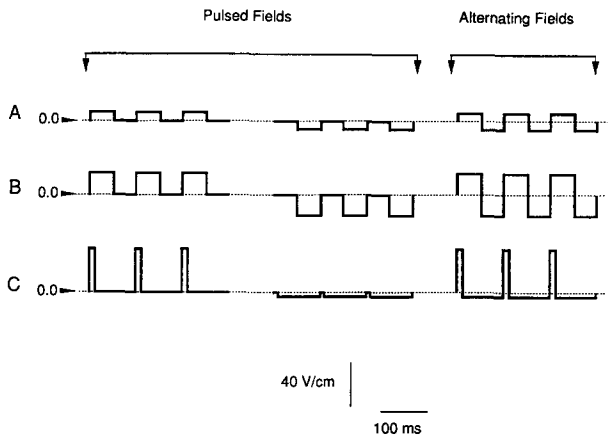

Figure 1. Schematic representation of the rapidly alternating electric field protocols used in this report. These waveforms were continuously applied to the cells, though only three cycles of each are shown. (*A*) The symmetric alternating field used in AChR studies, in which the field alternated between $^{+}8$ and 0, 0 and $^{-}8$, or $^{+}8$ and $^{-}8$ V/cm. (*B*) The symmetric alternating field used in fura-2 imaging studies, in which the field alternated between $^{+}20$ and 0, 0 and $^{-}20$, or $^{+}20$ and $^{-}20$ V/cm. The magnitude of these fields was increased to insure a reliably robust calcium signal. (*C*) The asymmetric alternating field used for both AChR and fura-2 studies, consisting of alternation between $^{+}40$ and 0, 0 and $^{-}4.4$, or $^{+}40$ $^{-}4.4$ V/cm. Each alternating (as opposed to pulsed) field has an average field strength of zero ($[field_1 \cdot duration_1] + [field_2 \cdot duration_2] = 0$). The pulsed fields used in AChR studies (*A* and *C*) are equivalent (average field strength = ± 4 V/cm).

Pulsed fields (monophasic waves, as opposed to the biphasic waves just discussed) were applied by inclusion of a diode (IN4005) in the circuit. AChR aggregation experiments were carried out using symmetric fields alternating between $^{+}8$ and $^{-}8$ V/cm. These fields were not sufficiently strong to induce a reliably robust calcium signal, so that fura-2 experiments with symmetric fields were performed with waveforms alternating between $^{+}20$ and $^{-}20$ V/cm. All experiments with asymmetric fields made use of waveforms alternating between $^{+}40$ and −4.4 V/cm.

Slowly reversing field paradigms consisted of fields alternating between $^{+}4$ and $^{-}4$ V/cm every 5–40 min. In each case the total time in the field was 80 min, and the experiment began one-quarter of the way through the cycle to avoid giving either cell pole a temporal advantage. For example the 40-min reversal protocol consisted of 20 min in one orientation followed by 40 min reversed, and then 20 min in the original orientation.

AChR Aggregation Experiments

For the AChR aggregation studies, cells were subjected to the indicated field paradigm and postfield relaxation period (if any). They were then chilled and incubated with 300 nM rhodamine-labeled α-bungarotoxin (prepared according to Ravdin and Axelrod, 1977) and 25 μg/ml Con A (Vector Laboratories, Inc., Burlingame, CA) for 10 min. Fluorescence video micrographs were collected by an RCA SIT video camera connected to a Zeiss Universal Microscope equipped with epi-illumination and a 40× Neofluor objective. The images were stored on video cassettes (models VO-5600 and VO-5800; Sony Umatic; Tokyo, Japan) and digitized on an IBM AT-compatible computer (American Micro Technology) equipped with a video digitizing board (model Digisector DS-88; The Microworks, Del Mar, CA). The digital images were corrected on a pixel-by-pixel basis for nonlinearities in the illumination, optics, and video camera. Pixels representing the cell perimeter (lying between 95 and 105% of the cell radius) were sorted into 16 sectors representing the angle from the left-facing cell pole. The intensities of the pixels in each sector were averaged to obtain an estimate of the distribution of fluorescently labeled receptors about the cell perimeter. Control experiments have confirmed the quantitative validity of this experimental approach (Stollberg and Fraser, 1988).

fura Experiments

fura-2/AM (Molecular Probes; Eugene, OR; ester form) stock solutions

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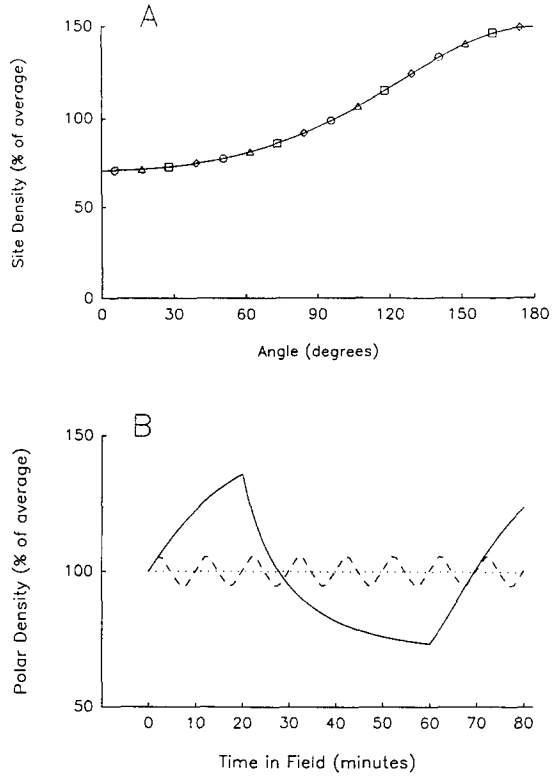

Figure 2. Simulations of ideally behaving sites in various electric field protocols. (*A*) The steady-state distribution of sites exposed for 80 min to field protocols having the same average field strength. (*Squares*) A constant field of 4 V/cm. (*Triangles*) A pulsed field paradigm in which the field strength alternates between 8 V/cm for 50 msec and 0 V/cm for 50 ms. (*Diamonds*) A pulsed field paradigm in which the field strength alternates between 40 V/cm for 10 ms and 0 V/cm for 90 ms. (*Circles*) A pulsed field paradigm in which the field strength alternates between 4.4 V/cm for 90 ms and 0 V/cm for 10 ms. The simulated distributions are identical, indicating that the sites behave according to the average field strength. (*B*) The polar density of sites exposed to symmetric alternating fields of the indicated period. The ordinate ("polar density") indicates the concentration of sites at the pole which starts off as the cathode; the anodal density is approximately mirror symmetric. (*Dotted line*) Field reversal every 50 ms (the reversal period for the rapidly alternating field experiments). The polar density remains unchanged at 100%. (*Dashed line*) Field reversal every 5 min. In this case the polar density never exceeds 106% of the average density. (*Solid line*) Field reversal every 40 min. The polar density increases to about 140% of the average value. See Materials and Methods for a description of the model and its assumptions.

were made by dilution of 50 μg into 50 μl pluronic f127 (Molecular Probes Inc., Eugene, OR) in dimethylsulfoxide, and were frozen in small aliquots for subsequent use. Loading buffer was made by a 1:500 dilution of the stock solution into Steinberg's solution containing 10 mM $Ca(NO_3)_2$. Cultures were gently rinsed several times in loading buffer, incubated for 30 to 60 min, and then rinsed in and returned to culture medium (all at room temperature in the dark). Cells were alternately illuminated at 345 and 375 nm ($\pm$6 nm) using a dual-monochromator, xenon arc light source (model Deltascan I; Photon Technology International; Princeton, NJ) and an electronically controlled shutter (Vincent Associates, Rochester, NY). Fluorescence imaging was performed on a Zeiss IM-35 inverted microscope using a 40× objective (model DApo 40 UV, 1.3 NA oil immersion; Olympus). Images were acquired with a SIT video camera (Hamamatsu Corp., Photonic Microscopy, Inc., Oak Brook, IL) connected to a VideoProbe image processor (ETM Systems, Mission Viejo, CA). After averaging 16 frames and subtracting backgrounds, the images at the two wavelengths were divided pixel-by-pixel (345 nm/375 nm) to form ratio images, which were stored for subsequent analysis.

Calibration of the fura imaging technique (Grynkiewicz et al., 1985) was accomplished by acquiring cell images at 345 and 375 nm illumination under the following incubation conditions: (*a*) 5 μM ionomycin with 0 Ca^{++}, 10 mM EGTA for 30 min, or (*b*) 1 μM ionomycin with 12 mM $Ca(NO_3)_2$ for 3–10 s. This procedure gave estimates for $Ratio_{min}$, $Ratio_{max}$, and a scaling factor K^*, which were used to convert ratio images to calcium images on a pixel-by-pixel basis using the equation

$$[Ca^{++}] = K^* \cdot (Ratio - Ratio_{min})/Ratio_{max} - Ratio).$$

Given the numerous assumptions underlying this equation and the methods used in estimating the required parameters, the true calcium concentrations may be significantly different from those calculated (see Almers and Neher, 1985 for discussion). For the purposes of this study these limitations are not important, as information about the relative changes in calcium concentration is all that is required.

To eliminate artifactually extreme values, which can occur at the cell boundaries of a calcium image, the pixels were median filtered using a 3 × 3 kernal (each pixel was assigned the median of nine values consisting of the pixel itself and its eight neighbors). Quantitative analysis of the subcellular distribution of calcium was performed on ratio images by breaking an annulus of pixels into 16 sectors as described for AChR aggregation studies. However, as the interest in these experiments is on intracellular (as opposed to surface) distributions, the annulus of pixels used was set to 85–95% of the cell radius, rather than the 95–105% annulus used in receptor aggregation studies.

Modeling of Site Behavior

Computer simulations were performed to determine the response of ideal sites to alternating electric fields ("ideal" sites are those which are affected only by electromigration and diffusion; see Stollberg and Fraser, 1990). The spherical cell surface was reduced to a one-dimensional string of 16 compartments, each representing one of the 16 sectors used in the video and analytical analyses. This dimensional simplification is made possible by the symmetry of the system, provided that the different surface areas and boundaries for each sector are taken into account. The electromigrational mobility, diffusion constant, and mobile fraction for the modeled sites were fixed at the measured values for Con A binding sites, as these are the best charac-

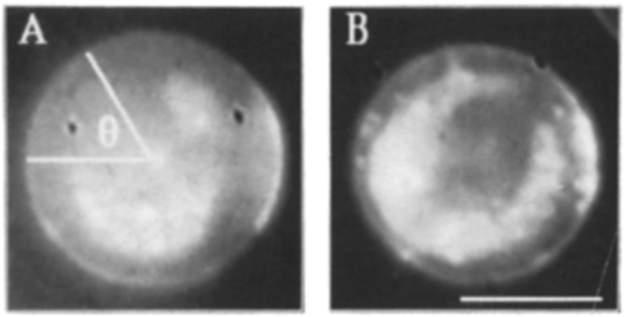

Figure 3. Videomicrographs of cultured *Xenopus* myospheres subjected to electric fields and labeled with fluorescent α-bungarotoxin to reveal the distribution of AChRs. These are examples of cells subjected to an average field strength of 4 V/cm for 20 min, followed by a 40-min postfield period. (*A*) A cell exposed to a constant field of 4 V/cm. (*B*) A cell exposed to a pulsed field alternating between 0 and 8 V/cm every 50 ms. Both images display autofluorescence inside the cell perimeter. In the examples shown this happens to be more noticeable in *B*, but there was no apparent difference in this feature (or any other) for most cells. The dark spots are the result of flaws in the video camera tube, and are excluded from consideration in the quantitative analysis of the images. A schematic on *A* summarizes the quantitative analysis performed on these and other images. The cell perimeter (95–105% of the radius) was scanned and grouped into 16 sectors representing the angle (Θ) from the left-facing cell pole (the anode), see Materials and Methods. Bar, 20 μm.

terized ideally behaving molecules (Stollberg and Fraser, 1988). At each iteration the flux equations for electromigration and diffusion were applied across the boundaries of the compartments. Integration was performed using the fourth-order Runge-Kutta method (Press et al., 1986). The validity of the simulation program was confirmed by comparison of simulated results with the analytical solutions for the distribution of sites at steady state (Jaffe, 1977), and for the decay from steady state in the absence of a field (Huang, 1973). In each case, the analytical and numerical approaches gave the same result to within <1%. The simulations presented in this publication were also checked by halving the time increment and doubling the number of sectors; this did not significantly alter the results. Simulations of slowly alternating electric fields were performed with the starting point one-quarter of the way through the waveform in order to model precisely the experimental manipulations described above.

Results

The experiments presented below depend on the use of time-varying electric fields (either pulsed or alternating fields) to distinguish between two possible mechanisms by which electric fields might trigger AChR clustering (see Jaffe and Nuccitelli, 1977). These possibilities are: (*a*) the electromigration-induced concentration of a non-AChR molecule, and (*b*) a voltage-sensitive mechanism (e.g., the activation of a voltage-gated calcium channel). These will be termed the electromigration and voltage-sensitive hypotheses, respectively. The redistribution of sites by electromigration is a slow (order of minutes) process that is linear with field strength and duration; that is, the flux of sites past a border is proportional to the field strength. The known voltage-sensitive mechanisms are fast (order of milliseconds) and nonlinear; examples are threshold events such as the firing of action potentials. These differences are used to test between the two hypotheses in the experiments presented below.

Because electromigration is a linear process, rapidly varying electric fields should produce the same effect as a constant electric field which is equal in magnitude to the average strength of the varying field. (In this context "rapidly" means rapid with respect to the several minutes required to change significantly the distribution of sites via electromigration.) To advance this argument beyond the intuitive level, simulations of the behavior of membrane molecules exposed to pulsed fields were performed (Fig. 2 *A*). The steady-state distribution of sites is shown for four different field protocols, each of which has an average field strength of 4 V/cm. The different protocols were also the same in terms of the rate of approach to steady state (result not shown). The excellent agreement of the simulations with each other, and with the analytically derived distribution of sites exposed to a constant electric field, illustrates dependence of electromigration on the average field strength (see Lin-Liu et al., 1984). It follows that any rapidly alternating field with an average field strength of 0 V/cm (e.g., the alternating field protocols used in this study) should produce no electromigration of sites. This understanding is confirmed by the simulation of ideally behaving sites in rapidly alternating electric fields (Fig. 2 *B*, *dotted line*; slowly alternating electric fields will be taken up at the end of this section.) Thus, the electromigration hypothesis makes a clear prediction with respect to rapidly alternating fields: they should not trigger AChR clustering.

If, on the other hand, receptor clustering is triggered by a voltage-sensitive mechanism, we would expect a different outcome in these alternating field protocols. Because voltage-sensitive mechanisms are nonlinear, the effects of a local depolarization during one phase of the field would not be nullified by the subsequent hyperpolarizations during the reverse phase. Thus, we would expect both poles of a cell to be activated by the rapidly alternating field protocols used in this study. Accordingly, the voltage-sensitive hypothesis makes a clearly distinct prediction with respect to rapidly alternating fields: they should trigger AChR clustering at both cell poles.

The first experimental question is whether pulsed and constant fields which are electromigrationally equivalent (i.e., have the same average field strength) are equally effective at triggering receptor clustering. To address this question, cells were subjected to a constant field, or a pulsed field protocol, each having an average field strength of 4 V/cm (see Fig. 1 for description of field protocols). Fig. 3 shows typical fluorescently labeled AChR images of cells placed in the constant field (Fig. 3 *A*), and the pulsed field (Fig. 3 *B*). As seen qualitatively, and in the quantitative analyses of many such cells, the clustering of AChRs proceeds in an indistinguishable manner for the two kinds of field paradigms. Quantitative analyses of the distribution of AChRs following pulsed fields are shown in Fig. 4 *A* (*top*). Not surprisingly, receptor clustering can be initiated at either the left or the right pole of the cell, depending on the orientation of the pulses.

Similar field protocols were used to assess the voltage-gated calcium response, as measured by fura-2 imaging (Grynkiewicz et al., 1985). Individual examples of the calcium distribution in muscle cells placed in pulsed electric fields are shown in Fig. 5, *B* and *C*; quantitative analyses are shown in Fig. 4 *B*. The concentration of intracellular calcium increases at the region of receptor clustering in this field paradigm (compare Fig. 4, *A* and *B*, *top*), as in the case of constant electric fields (result not shown). These increases followed the imposed electric fields in less than 2 s (the limit of temporal resolution available with the imaging techniques used). As this is much too fast for an electromigrational effect, the calcium response is clearly due to the depolarization of the cell membrane and the subsequent action of voltage-gated channels. Fig. 4, *A* and *B* (*top*) show that receptor clustering and local calcium levels are correlated in these pulsed field protocols, but this of course does not demonstrate that the increased calcium levels cause receptor clustering.

The lower panels of Fig. 4 show the resulting receptor distribution (Fig. 4 *A*) and calcium concentration (Fig. 4 *B*) for lalternating electric fields (the sum of the two pulsed field protocols just discussed). This experiment clearly shows that the correlation between receptor clustering and intracellular calcium breaks down. The calcium distribution is exactly what is expected of the nonlinear response characteristic of voltage-sensitive mechanism; the increase in calcium due to depolarization is not nullified by alternating hyperpolarizations of equal duration and magnitude. The result is that local calcium concentrations are raised at both cell poles (Fig. 4 *B*, *bottom*; Fig. 5 *D*). If receptor clustering is triggered by a local rise in calcium, or by the action of some other nonlinear mechanism, the cells should show receptor clusters at both cell poles under these conditions. Instead, the receptors are not clustered at either cell pole (Fig. 4 *A*, *bottom*). This is the result expected if the cluster triggering event is dependent on a slow (requiring minutes) linear process such as elec-

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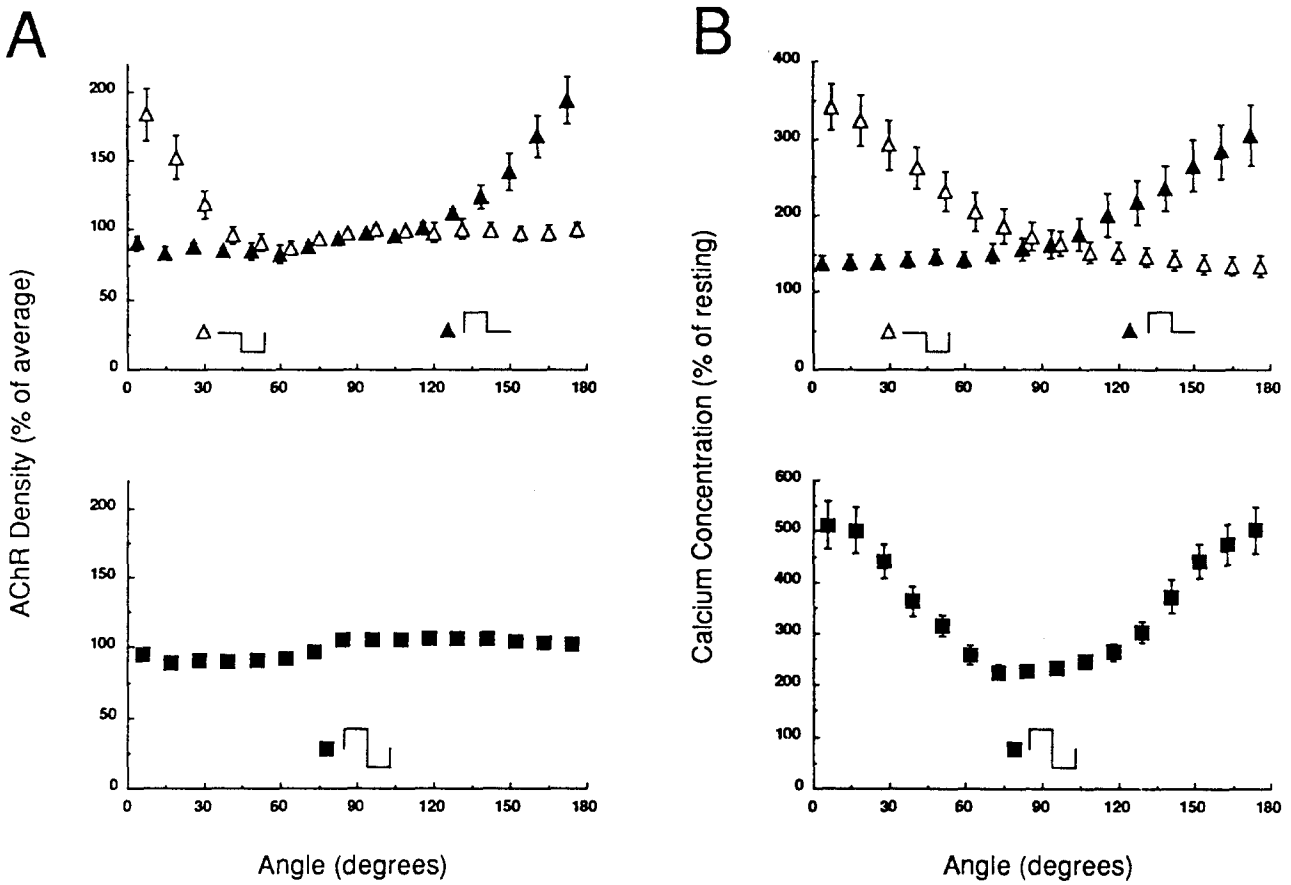

Figure 4. Quantitative analysis of the distribution of AChRs (*A*) and intracellular calcium (*B*) on myospheres subjected to the indicated electric field protocols. (*Solid* and *open triangles*) The individual pulsed fields which together comprise the symmetric alternating field. (*Solid squares*) The symmetric alternating field. Receptor clustering and intracellular calcium concentrations are correlated with either half of the alternating electric field, but not correlated for the alternating field itself (bottom panels). In the case of AChR experiments (*A*), cells were exposed to the indicated field paradigm for 20 min, followed by a 40-min postfield period, and were then labeled and analyzed. The images used to calculate the calcium distributions (*B*) were acquired during the application of the indicated field protocol. See Fig. 1 for detailed description of the field protocols. Note the difference in ordinates for the top and bottom panels of *B*.

tromigration. This result favors the electromigration hypothesis, and clearly indicates that a local increase in intracellular calcium is not sufficient to induce receptor clustering.

Asymmetric Alternating Fields

Another approach to testing between the electromigration and voltage-sensitive mechanisms of receptor clustering involves the use of asymmetric alternating fields (Cooper et al., 1989). These are fields in which a brief, large magnitude field is followed by a longer duration, smaller magnitude field (Fig. 1 *C*). In the experiments described here, values for the durations and magnitudes of the two phases of the waveform were chosen so that the average field strength was 0 V/cm (see Fig. 1 *C*). In other words, the two phases of the alternating waveform are equivalent in terms of electromigration. However, they would not be expected to be equivalent in stimulating any process which has a nonlinear response. In the case of voltage-gated channels, for example, a depolarization of 50 mV for 10 ms would be expected to be more effective than one of 10 mV for 50 ms.

The receptor clustering responses to the pulsed and asymmetric field paradigms are shown in Fig. 6 *A*, and subcellular calcium distributions of cells exposed to the same field paradigms are shown in Fig. 5 and 6 *B*. The brief, high magnitude pulsed field train is clearly sufficient to trigger receptor clustering (Fig. 6 *A*, *top*). The same protocol also induces a local increase in calcium (Fig. 5 *E*; Fig. 6 *B*, *top*). Thus, receptor clustering is correlated with a local rise in intracellular calcium. This is consistent with either of the experimental hypotheses (electromigration or voltage-sensitive triggering of receptor clustering), and therefore does not distinguish between them.

A clear test of mechanism is available from the effects of the long, low magnitude pulsed field. This protocol triggers receptor clustering to the same extent as the brief, high magnitude pulsed field train (Fig. 6 *A*, *top*). This is as expected if the trigger stimulus is electromigration, as the two protocols have the same average field strength. The equivalence would not be expected of a voltage-sensitive mechanism, however, and is not in fact seen in the distribution of intracellular calcium. Instead, the long, low magnitude pulse train has no discernable effect on intracellular calcium levels (Fig. 5 *F* vs. 5 *E*; Fig. 6 *B*, *top*). This is another clear example in which receptor clustering and intracellular calcium levels are not correlated. This result suggests that a local increase in intracellular calcium is not necessary for the induction of receptor clustering (see also Discussion).

In the final set of experiments, the cells were subjected to

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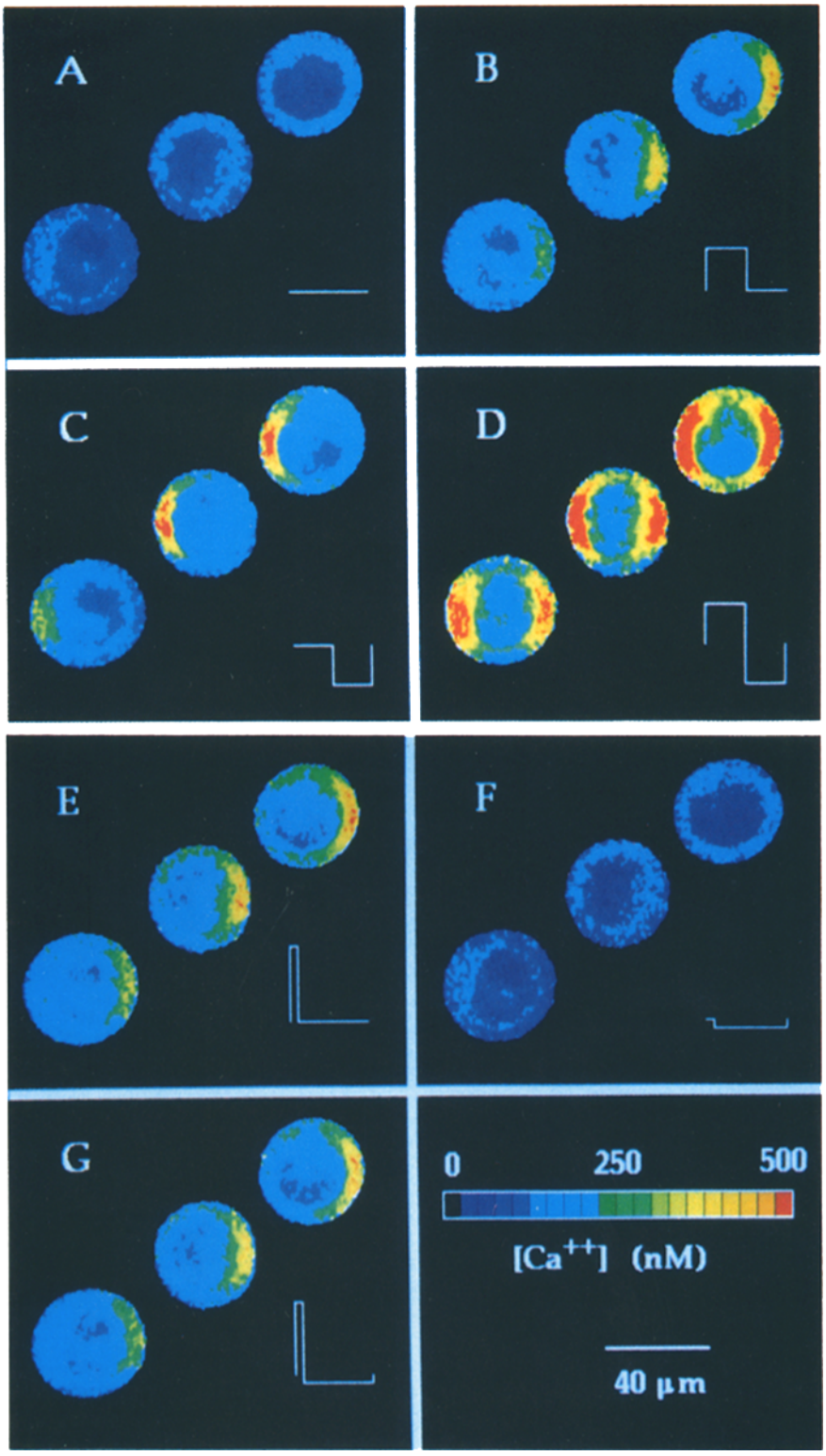

Figure 5. Pseudocolor representations of intracellular calcium levels in cells subjected to different field paradigms. *A–G* depict the same three cells. (*A*) No field. (*B* and *C*) The individual pulsed fields that together comprise the alternating field (*D*). *B* and *C* clearly show polar increases in calcium, while *D* displays a bipolar increase. (*E* and *F*) The individual pulsed fields that together comprise the asymmetric alternating field (*G*). The short, high magnitude pulses in *E* clearly raise the polar calcium concentration, while the longer, lower magnitude pulses in *F* have no discernable effect (compare with *A*). The asymmetric alternating field (*G*) increases the polar calcium concentration in a manner quite similar to the short, high magnitude pulsed field alone (*E*). These were the only cells within the microscopic field of view during this experiment; the cell images were digitally translocated to make more efficient use of space in the figure. See Fig. 1 for detailed description of the field protocols. Note the caveats in Materials and Methods regarding calibration of absolute calcium concentration. The true values may differ from those indicated here, but the relative changes in calcium concentration may be relied upon. This figure was generated using the public domain program "Image" (v. 1.23h) by Dr. Wayne Rasband of the National Institutes of Health, Bethesda, MD.

an asymmetric alternating field made up of the two pulsed field protocols just discussed. The two phases of the waveform cancel each other out in terms of receptor clustering, resulting in a uniform distribution of receptors (Fig. 6 *A*, *bottom*). This is the outcome predicted by the electromigration hypothesis, as there should be no net accumulation of membrane molecules under these conditions. The calcium distribution does not correlate with receptor clustering, and instead reveals a response very similar to that seen with the brief, high-magnitude pulsed field (Fig. 5 *E* versus *G*; Fig. 6 *B*). Once again, this is precisely the behavior to be expected from a nonlinear process, and is striking in its lack

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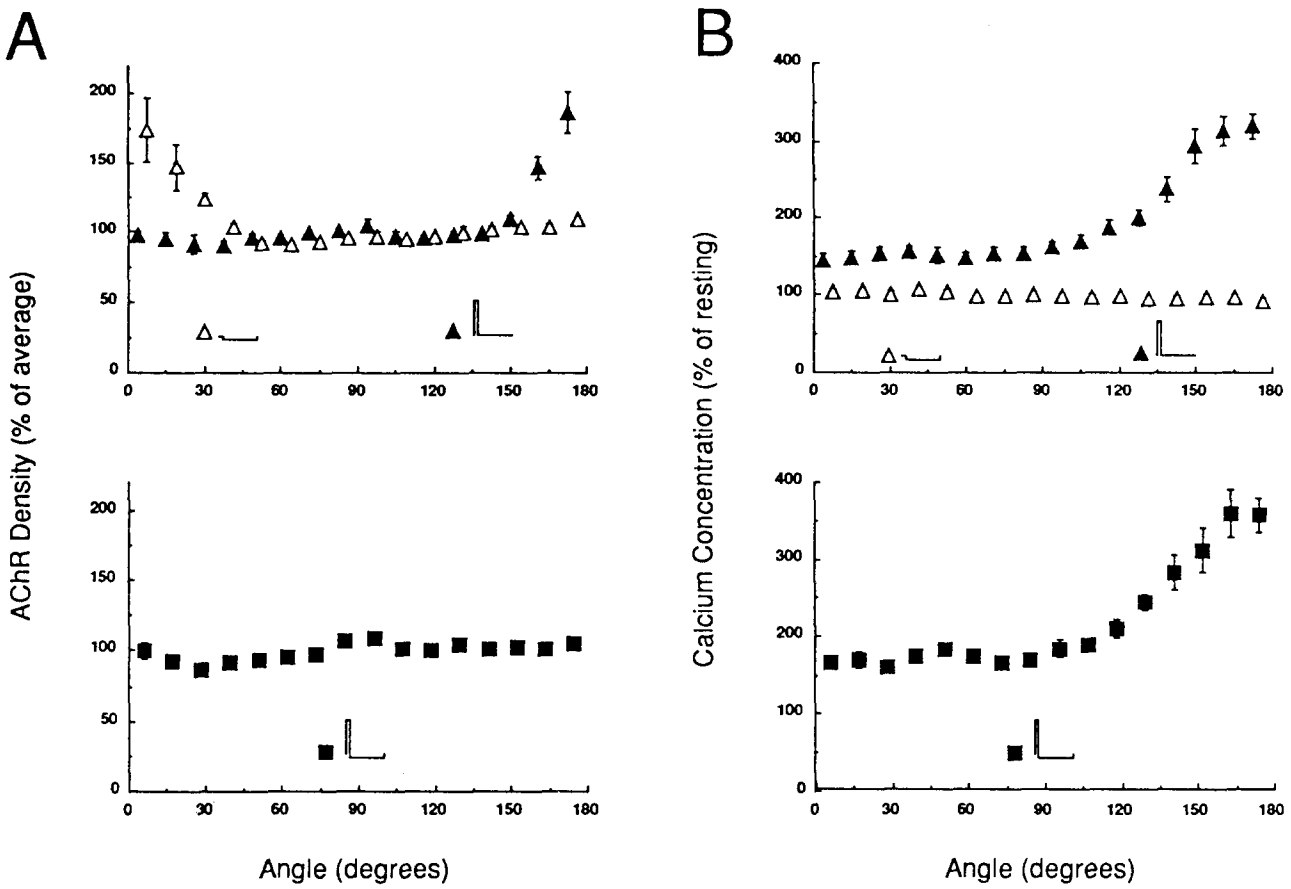

Figure 6. Quantitative analysis of the distribution of AChRs (*A*) and intracellular calcium (*B*) on myospheres subjected to the indicated electric field protocols. (*Solid* and *open triangles*) The individual pulsed fields that together comprise the asymmetric alternating field. Receptor clustering and increased polar calcium are correlated for the brief, high magnitude pulsed field (*solid triangles*). The longer, low magnitude pulses result in receptor clustering, but no detectable increase in polar calcium (*open triangles*). The asymmetric alternating field does not trigger receptor clustering, but does increase the calcium concentration at one pole. In the case of AChR experiments (*A*), cells were exposed to the indicated field paradigm for 20 min, followed by a 40-min postfield period, and were then labeled and analyzed. The images used to calculate the calcium distributions (*B*) were acquired during the application of the indicated field protocol. See Fig. 1 for detailed description of the field protocols.

of correlation with receptor clustering (Fig. 6 *A*, *bottom* versus 6 *B*, *bottom*). As in the result using symmetrically alternating fields, this observation rules out the possibility that locally increased calcium levels are sufficient to trigger receptor clustering.

Slowly Alternating Fields

Thus far we have presented data in which the predictions of a voltage-sensitive mechanism are not in general satisfied. An additional manipulation was used to examine the predictions of the electromigration hypothesis. It was argued above that rapidly alternating symmetric fields should have no net effect on the electromigration of membrane molecules. This argument can be presented qualitatively: before significant electromigration can proceed toward one cell pole, the field (and hence the electromigrational flux) reverses, cancelling out the flux in the first direction. If the alternation were slowed sufficiently, however, one would expect that the cluster triggering molecule might accumulate to a significant extent at one cell pole before field reversal led to its concentration at the opposite cell pole. In this scenario, slowly alternating symmetric fields would be expected to give rise to cells having receptor clusters at both cell poles.

To test this argument quantitatively, simulations of the behavior of membrane molecules exposed to alternating fields were performed. The putative molecule which triggers receptor clustering is assumed to behave ideally (solely in response to electromigration and diffusion) at least up to the point at which it triggers receptor aggregation. The simulated polar densities of these sites are shown in Fig. 2 *B* as functions of time for fields reversing at three different rates. As mentioned previously, rapidly alternating electric fields result in no net migration of sites (Fig. 2 *B*, *dotted line*). As expected, slowing down the alternation period results in larger swings in the polar density of electromigrating molecules. However, even reversal periods as long as 5 min result in <6% change in the polar density (Fig. 2 *B*, *dashed line*). When the reversal period has been slowed to 40 min, the polar density is raised to ~140%, reaching ~75% of the value produced by a constant field of the same magnitude (4 V/cm). Thus, the electromigration hypothesis predicts that slowly alternating fields (reversals on the order of 10's of minutes) will trigger receptor clustering at both cell poles.

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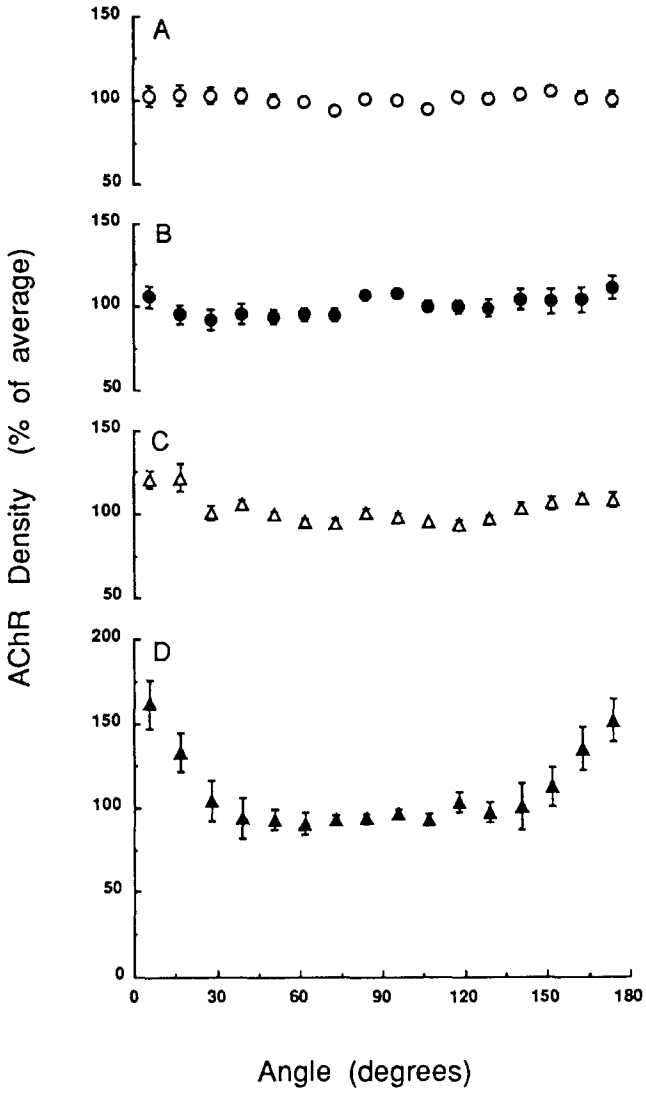

Figure 7. Quantitative analysis of the distribution of AChRs on myospheres subjected to slowly alternating electric fields. Cells were subjected for 80 min to symmetrically alternating electric fields with the same field strength (4 V/cm), but different reversal periods. (*A*) Reversal every 5 min. (*B*) Reversal every 10 min. (*C*) Reversal every 20 min. Note the nearly uniform distributions in *A–C*. (*D*) Reversal every 40 min. Note the bimodal distribution of receptors.

The results of slowly alternating field experiments are shown in Figs. 7 and 8. Receptor clustering was not observed with field reversal times of 5 or 10 min (Fig. 7, *A* and *B*). The receptor distribution after fields reversing every 20 min is essentially uniform, although there is the suggestion that perhaps the threshold for receptor clustering is nearly achieved (Fig. 7 *C*). When the reversal time is lengthened to 40 min, the cells clearly show a bimodal distribution of receptors (Fig. 7 *D*). Many of the cells in question show unambiguous receptor clustering at both cell poles (Fig. 8, *A* and *B*), in clear agreement with the predictions of the electromigration hypothesis.

Discussion

Electric fields do not simply cluster AChRs, but rather induce or trigger a receptor clustering event (Stollberg and Fraser, 1988, 1989, 1990). The distinction emphasized here is that clustering continues (polar AChR density continues to increase) after termination of the field. Field-induced receptor clustering appears to take place by way of a diffusion trap. The trapping mechanism is somewhat specific for AChRs in that the heterogeneous Con A binding sites are not clustered (Stollberg and Fraser, 1988). Due to the simplicity of this model system, there are only three known mechanisms by which receptor clustering could be initiated. Of these, the simplest possibility is that the triggering event for receptor clustering is a local increase in receptor density. This hypothesis has been ruled out by documenting conditions under which receptor electromigration proceeds toward the anodal pole, while aggregation continues to occur at cathodal cell pole (Stollberg and Fraser, 1989, 1990). This means that receptor clustering requires the involvement of a nonreceptor molecule.

This leaves two remaining experimental hypotheses that can account for field-induced receptor clustering, and the experiments presented here are designed to test between these two alternatives. One possibility is that some membrane-associated molecule is concentrated at the cathodal cell pole by electromigration, and that its increased density triggers AChR aggregation. A second possibility is that the depolarization of the cell membrane at the cathodal pole triggers receptor aggregation. Given the role of calcium in intracellular signaling and the dependence of receptor clustering on external calcium levels (see below), a voltage-gated calcium channel would be an attractive candidate for the mediation of receptor clustering. The two hypotheses make many similar experimental predictions, but they differ in one important respect. Electromigration is a slow (order of minutes) process that is linear with field strength and duration, whereas all known voltage-sensitive mechanisms are fast (order of milliseconds) and nonlinear. Consequently the clustering behavior of AChRs subjected to time-varying fields depends on whether the trigger is electromigration or a voltage-sensitive mechanism (Jaffe and Nuccitelli, 1977).

Because electromigration is a linear process, rapidly pulsed or alternating fields are electromigrationally equivalent to constant fields having the save average field strength. This can be seen clearly in the simulated behavior of sites in response to the different field protocols shown in Fig. 2 *A*. The rapidly alternating (as opposed to pulsed) fields used in the present work have in common an average field strength of 0 V/cm, and accordingly should not be effective as electromigrational stimuli. In qualitative terms, this is because any electromigration occurring during one phase is "undone" in the subsequent phase. This intuitive analysis is confirmed by the uniform distribution of (*a*) simulated ideally behaving sites (Fig. 2 *B*, *dotted line*), and (*b*) Con A binding sites (data not shown) in response to rapidly alternating fields. Accordingly, the electromigration hypothesis predicts that these fields will not induce AChR clustering. This is exactly what was found: the pulsed fields individually trigger receptor clustering, but when summed to produce the alternating field the result is a uniform distribution of receptors (Fig. 4 *A*, *top* versus *bottom*). This outcome would not be expected from the voltage-sensitive hypothesis, because nonlinearity guarantees that whatever signal arises from a depolarizing pulse is not "undone" by a hyperpolarizing pulse of equal magnitude. Thus, the voltage-sensitive hypothesis predicts bipo-

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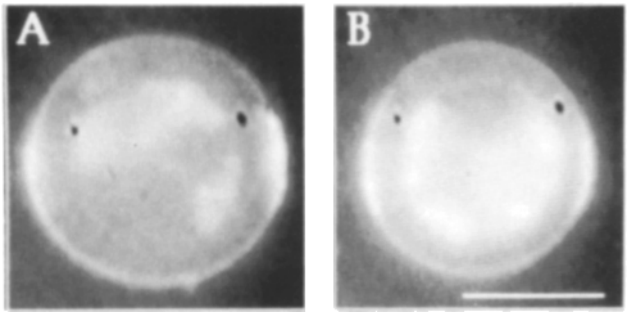

Figure 8. Videomicrographs of myospheres subjected to slowly alternating electric fields and labeled with fluorescent α-bungarotoxin to reveal the distribution of AChRs. These are examples of cells subjected to an electric field consisting of +4 V/cm for 20 min, −4 V/cm for 40 min, and +4 V/cm for 20 min. Note the receptor clusters at both cell poles. Bar, 20 μm.

lar receptor clusters under these conditions. In support of this view, we find that intracellular calcium levels do in fact show a bipolar distribution in response to this alternating field paradigm (Fig. 4 *B*, *bottom*; Fig. 5 *D*).

The two hypotheses also make unique predictions about the effects of asymmetric alternating fields (Cooper et al., 1989). The pulsed field protocols used to generate the asymmetrically alternating fields are electromigrationally equivalent, as they have the same average field strength (see Fig. 1 *C*). Thus, the asymmetric field has an average field strength of 0 V/cm. The predictions of the electromigration hypothesis are therefore (*a*) the long, low magnitude pulses should be as effective at triggering AChR clustering as the brief, high magnitude pulses, and (*b*) the asymmetric alternating field should not trigger receptor clustering. Both predictions are born out by the data: the two pulsed fields caused similar amounts of receptor clustering (Fig. 6 *A*, *top*), and together in the asymmetric alternating field they caused no receptor clustering (Fig. 6 *A*, *bottom*).

The voltage-sensitive hypothesis, on the other hand, predicts that (*a*) the brief, high magnitude pulses should be more efficacious than the long, low magnitude pulses in terms of receptor clustering, and (*b*) that the alternating field should trigger monopolar clusters much as the short, high magnitude pulsed field. This analysis is supported by our observations of the subcellular calcium distributions in cells exposed to these field protocols. The brief, high magnitude pulses stimulated calcium levels, but the long, low magnitude pulses had no discernable effect (Fig. 5 *E* versus *F*; Fig. 6 *B*, *top*). Furthermore, when the two protocols are combined to form the asymmetric alternating field, the result is a calcium response indistinguishable from the brief, high magnitude pulse train (Fig. 5 *G* versus *E*; Fig. 6 *B*, *top* versus *bottom*).

These observations clearly indicate that a local increase in intracellular calcium levels is not sufficient to trigger receptor clustering. Both symmetric and asymmetric alternating fields can produce locally increased calcium levels without triggering receptor clustering. The evidence also suggests that a local increase in the concentration of calcium is not necessary for receptor clustering. The long duration, low magnitude half of the asymmetric wave form induces receptor clustering, but results in no detectable increase in intracellular calcium (Fig. 5, *A* versus *F*). Although an increase below the threshold for detection could have occurred, it seems unlikely that such a small change (<10% of the resting level) could be an absolute requirement for AChR clustering.

Numerous reports suggest that receptor clustering is at least partially blocked by removal of extracellular calcium whether the clustering stimulus is a neurite (Henderson et al., 1984; Davey and Cohen, 1986), positively charged latex beads (Peng, 1984; Zhu and Peng, 1988), agrin (Wallace, 1988), or electric fields (Stollberg and Fraser, 1988, 1989). How can these reports be reconciled with the interpretation put on the experiments presented here? The resolution of this apparent conflict involves a distinction not previously made between two ways in which calcium might be required for receptor clustering. One possibility is that the normal resting concentration of calcium (either intra- or extracellular) is required for receptor clustering. Such a requirement could arise, for example, from a role for calcium-dependent binding in AChR cluster formation (Takeichi, 1977; Grunwald et al., 1980; Brackenbury et al., 1981). This possibility need not require a local increase in intracellular calcium to trigger receptor clustering, and can be characterized as representing a permissive requirement for calcium. Removal of extracellular calcium could well result in calcium levels (intra- or extracellular) that are below the required threshold. Thus the partial blockade of receptor clustering in the absence of external calcium can be interpreted as evidence for a permissive role for calcium. A second possibility would be that a local increase in calcium is required for receptor clustering, or in other words that calcium levels play an instructive role in triggering AChR clustering. Our present results and the findings cited above can be reconciled by characterizing the role of calcium as permissive, rather than instructive, with respect to receptor clustering. This rules out a class of otherwise attractive hypotheses about the role of calcium in receptor clustering.

The logic underlying these experiments can readily be extended to exclude other instances of a voltage-sensitive mechanism. All known voltage-gated channels behave non-linearly, and are therefore unlikely to account for the receptor clustering results summarized above. This interpretation is supported by the observation that field-induced receptor clustering is unaffected by the removal of extracellular sodium (result not shown), demonstrating the independence of receptor clustering on a voltage-gated sodium flux. Thus, voltage-sensitive mechanisms are not sufficient, and probably not necessary, for the induction of AChR clusters. Having ruled out the alternatives, it appears that receptor clustering is triggered by a local increase (or possibly a decrease) in the density of some non-AChR molecule.

This possibility is supported by the final set of experiments, in which slowly alternating fields were applied. In these experiments the fields were reversed every 5–40 min, while the field magnitude and the duration of the experiment were held constant. The electromigration hypothesis predicts that if the reversal period is lengthened sufficiently, receptor clustering should be induced at both cell poles. Simulation of this behavior suggests that the critical reversal period would likely be between 5 and 40 min (Fig. 2). The critical reversal period cannot be estimated precisely without knowing the diffusion constant and threshold density of the molecule which triggers receptor clustering. However, any reasonable assumptions about these parameters support the prediction that the reversal period would have to be on the

order of minutes to 10's of minutes to induce double clustering. In concurrence with this prediction, receptor clustering was found to proceed at both cell poles when the fields were alternated every 40 min, but not when reversed at 5-, 10-, or 20-min intervals (Figs. 7 and 8). This finding rules out a fast, voltage-sensitive mechanism as the trigger for receptor clustering, but one could still account for the result if there were a slow, intermediate step between channel activation and the clustering of receptors. However, this interpretation is inconsistent with results from the rapidly alternating field experiments discussed above. Together, therefore, these observations make a strong case for the electromigration hypothesis: the local change in the density of some as yet unidentified molecule (not the AChR) triggers AChR clustering.

What can be said about the properties of this molecule that triggers receptor clustering in response to external electric fields? The triggering molecule must of course be mobile in the plane of the membrane, and it must extend into the extracellular domain. This latter important restriction arises out of the insulating properties of the cell membrane. As a consequence of this insulating barrier, these small, spherical cells are isopotential, which is another way of saying that the field strength inside the cell is essentially zero (this need not be the case in vivo: see Stollberg and Fraser, 1989; Poo and Young, 1990). Because of this, only molecules with at least part of their structure extending into the cell exterior will move in response to the field. It seems clear, therefore, that the molecule in question is an integral or (extracellular) peripheral membrane component, or a part of the extracellular matrix.

Our results suggest, but do not prove, that the primary clustering event in vivo is the localization of membrane-associated components. This is clearly true for field-induced receptor clusters, and parsimony suggests that it may be true more generally. Certainly it is clear that the signal in vivo cannot be a local increase in calcium concentration, or the action of a voltage-gated channel, as these are shown in the present study to be insufficient to trigger receptor clustering. Although various intracellular components are clearly associated with receptor clusters induced by neuritic or other contacts (Bloch and Pumplin, 1988), and indeed by electric fields (Rochlin and Peng, 1989), the role of these components in the initiation of receptor clustering has yet to be demonstrated. The localization of cytoskeletal components is unlikely to be the initial triggering event in vivo either, as the signal from a neurite must be transmitted across the cell membrane in some way (see below). Given our present results, the most likely triggering event for the organization of cytoplasmic components is the local concentration of an integral membrane protein. The local concentration of such molecules would increase the effective valency of cytoplasm-facing sites that bind to intracellular components, thereby increasing their binding avidity.

The molecule responsible for cytoskeletal organization could be the same molecule that triggers field-induced receptor clustering, or another molecule concentrated as a consequence of the cluster-initiating molecule. An interesting possibility is that cytoskeletal elements are organized by the AChR itself, further stabilizing the growing receptor cluster. Even if the initial organization of intracellular components is caused by (rather than causes) the localization of integral membrane components, it could be that cytoskeletal attachments form an important link in a positive feedback loop which increases the efficiency (or permanence) of receptor aggregates. It could also be that intracellular binding events are necessary for initial receptor clustering. This possibility is complicated by the observation that mild proteolytic digestion blocks field-induced receptor clustering, and breaks down preformed receptor clusters (Stollberg and Fraser, 1988). Thus, it seems that at least one binding event which is necessary for receptor clustering takes place on the extracellular surface.

A final question concerns the mechanism by which a neurite communicates the signal responsible for initiating AChR clustering to the muscle cell. There are three proposed mechanisms by which the neurite could in principle transmit this signal: by the release of neurite-derived factors (Godfrey et al., 1984; Usdin and Fischbach, 1986), by contact mediated events (Bloch and Pumplin, 1988; Peng et al., 1981), or by the endogenous electric fields created by local release of acetylcholine (Fraser and Poo, 1982; Stollberg and Fraser, 1989). Of course these are not mutually exclusive possibilities. Any one of these mechanisms (or a combination) could reasonably be expected to raise the local concentration of specific membrane-associated molecules, thereby initiating the receptor clustering event. The molecule that is defined by electric field experiments as the primary causal factor in initiating AChR clustering has yet to be identified, but the experimental system is well suited to screening likely candidates.

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